(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 August 2001 (23.08.2001)

PCT

(10) International Publication Number
WO 01/61017 A2

(51) International Patent Classification[7]: C12N 15/62, 15/28, C07K 14/525, 14/57, A61K 38/19, 38/21, C12N 15/23, A61K 31/70, C07K 16/28, 5/08, 7/00, 5/093

(21) International Application Number: PCT/EP01/01543

(22) International Filing Date: 13 February 2001 (13.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
MI2000A000249 15 February 2000 (15.02.2000) IT

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/61017 A2

(54) Title: MODIFIED CYTOKINES FOR USE IN CANCER THERAPY

(57) Abstract: Cytokine derivatives capable of homing the tumoral vessels and the antigen presenting cells and the use thereof as antitumoral agents.

MODIFIED CYTOKINES FOR USE IN CANCER THERAPY

The present invention refers to modified cytokines for use in the treatment of cancer. More particularly, the invention refers to cytokines derivatives capable of "homing" tumor vessels and antigen presenting cells.

The antitumoral activity of some cytokines is well known and described. Some cytokines have already been used therapeutically also in humans *(29)*. For example, such cytokines as interleukine-2 (IL-2) and interferon α(IFNα) have shown positive antitumoral activity in patients with different types of tumors, such as kidney metastatic carcinoma, hairy cell leukemia, Kaposi sarcoma, melanoma, multiple mieloma, and the like. Other cytokines like IFNß, the Tumor Necrosis Factor (TNF) α, TNFß, IL-1, 4, 6, 12, 15 and the Colony Stimulating Factors (CFSs) have shown a certain antitumoral activity on some types of tumors and therefore are the object of further studies.

In general, the therapeutic use of cytokines is strongly limited by their systemic toxicity. TNF, for example, was originally discovered for its capacity of inducing the hemorrhagic necrosis of some tumors *(1)*, and for its in vitro cytotoxic effect on different tumoral lines *(2)*, but it subsequently proved to have strong pro-inflammatory activity, which can, in case of overproduction conditions, dangerously affect the human body *(3)*.

As the systemic toxicity is a fundamental problem with the use of pharmacologically active amounts of cytokines in humans, novel derivatives and therapeutic strategies are now under evaluation, aimed at reducing the toxic effects of this class of biological effectors while keeping their therapeutic efficacy.

Some novel approaches are directed to:

a) the development of fusion proteins which can deliver TNF into the

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tumor and increase the local concentration. For example, the fusion proteins consisting of TNF and tumor specific-antibodies have been produced *(4)*;

b) the development of TNF mutants which maintain the antitumoral activity and have a reduced systemic toxicity. Accordingly, mutants able of selectively recognizing only one receptor (p55 or p75) have been already prepared *(5)*;

c) the use of anti-TNF antibodies able to reduce some toxic effects of TNF without compromising its antitumoral activity. Such antibodies have been already described in literature *(30)*;

d) the use of TNF derivatives with a higher half-life (for example TNF conjugated with polyethylene glycol).

The preparation of TNF derivatives capable of selectively targeting the tumoral sites has been recently reported. For example, a fusion protein has been described, obtained by fusing the gene of the heavy chain of an anti-transferrin receptor mAb and the TNF gene *(4)*, or a fusion protein of TNF with the "hinge" region of a monoclonal antibody against the tumor-associated TAG72 antigen *(6)*, or a Fv-TNF fusion protein (*6*).

EP 251 494 discloses a system for administering a diagnostic or therapeutic agent, which comprises: an antibody conjugated with avidin or streptavidin, an agent capable of complexing the conjugated antibody and a compound consisting of the diagnostic or therapeutic agent conjugated with biotin, which are administered sequentially and adequately delayed, so as to allow the localization of the therapeutic or diagnostic agent through the biotin-streptavidin interaction on the target cell recognized by the antibody. The described therapeutic or diagnostic agents comprise metal chelates, in particular chelates of radionuclides and low molecular weight antitumoral agents such as cis-platinum, doxorubicin, etc.

EP 496 074 discloses a method which provides the sequential administration of a biotinylated antibody, avidin or streptavidin and a biotinylated diagnostic or therapeutic agent. Although cytotoxic agents like ricin are generically mentioned, the application relative to radiolabelled compounds is mostly disclosed.

WO 95/15979 discloses a method for localizing highly toxic agents on cellular targets, based on the administration of a first conjugate comprising the specific target molecule conjugated with a ligand or an anti-ligand followed by the administration of a second conjugate consisting of the toxic agent bound to an anti-ligand or to the ligand.

WO 99/13329 discloses a method for targeting a molecule to tumoral angiogenic vessels, based on the conjugation of said molecule with ligands of NGR receptors. A number of molecules have been suggested as possible candidates, but doxorubicin only is specifically described. No use of ligands of NGR receptors as cytokines vehicles to induce immuno responses is disclosed.

It has now surprisingly been found that the therapeutic index of certain cytokines can be remarkably improved and their immunotherapeutic properties can be enhanced by coupling with a ligand of aminopeptidase-N receptor (CD13). CD13 is a trans-membrane glycoprotein of 150 kDa highly conserved in various species. It is expressed on normal cells as well as in myeloid tumor lines, in the angiogenic endothelium and is some epithelia. CD13 receptor is usually identified as "NGR" receptor, in that its peptide ligands share the amino acidic "NGR" motif.

According to a first aspect, the invention provides a conjugation product of a cytokine selected from TNF and IFNγ and a ligand of CD13 receptor. Said ligand can be an antibody or a fragment thereof such as Fab, Fv, single-chain Fv, a peptide or a peptido-mymetic, namely a peptido-like

molecule capable to bind the CD13 receptor, optionally containing modified, not naturally occurring amino acids. The ligand is preferably a straight or cyclic peptide comprising the NGR motif, such as CNGRCVSGCAGRC, NGRAHA, GNGRG, cycloCVLNGRMEC or cycloCNGRC, or, more preferably, the peptide CNGRC. The peptide can be coupled directly to the cytokine or indirectly through a spacer, which can be a single amino acid, an amino acid sequence or an organic residue, such as 6-aminocapryl-N-hydroxysuccinimide. The coupling procedures are known to those skilled in the art and comprise genetic engineering or chemical synthesis techniques.

The peptide ligand preferably is linked to the cytokine N-terminus thus minimizing any interference in the binding of the modified cytokine to its receptor. Alternatively, the peptide can be linked to amino acid residues which are amido- or carboxylic- bonds acceptors, naturally occurring on the molecule or artificially inserted with genetic engineering techniques. The modified cytokine is preferably prepared by use of a cDNA comprising a 5'-contiguous sequence encoding the peptide.

According to a preferred embodiment, there is provided a conjugation product between TNF and the CNGRC sequence. More preferably, the amino-terminal of TNF is linked to the CNGRC peptide through the spacer G (glycine).

The resulting product (NGR-TNF), proved to be more active than TNF on RMA-T lymphoma animal models. Furthermore, animals treated with NGR-TNF were able to reject further tumorigenic doses of RMA-T or RMA cells. The increase in the antitumoral activity, as compared with normal TNF, could be observed in immunocompetent animals but not in immunodeficient animals. This indicates that the increase in the antitumoral activity of TNF conjugated with "NGR" peptides is due to an enhanced

immune response rather than to a direct cytotoxic activity of the conjugate.

It has also been demonstrated that the *in vivo* immune effects induced by NGR-TNF are directly related to the CD13 receptor. It has, for example, been observed that antibody against the CD13 receptor as well as the GNGRC ligand compete with NGR-TNF *in vivo*, thus suggesting a mechanism of receptor *targeting* by NGR-TNF.

The therapeutic index of the TNF/ CD13 ligand conjugates can be further improved by using a mutant form of TNF capable of selectively binding to one of the two TNF receptors, p75TNFR and p55TNFR. Said TNF mutant can be obtained by site-directed mutagenesis *(5; 7)*.

The pharmacokinetic of the modified cytokines according to the invention can be improved by preparing polyethylene glycol derivatives, which allow to extend the plasmatic half-life of the cytokines themselves.

A further embodiment of the invention is provided by bifunctional derivatives in which the cytokines modified with the CD13 ligand are conjugated with antibodies, or their fragments, against tumoral antigens or other tumor angiogenic markers, e.g. αv integrins, metalloproteases or the vascular growth factor, or antibodies or fragments thereof directed against components of the extracellular matrix, such as anti-tenascin antibodies or anti-fibronectin EDB domain. The preparation of a fusion product between TNF and the hinge region of a mAb against the tumor-associated TAG72 antigen expressed by gastric and ovarian adenocarcinoma has recently been reported (6).

A further embodiment of the invention is provided by the tumoral pre-targeting with the biotin/avidin system. According to this approach, a ternary complex is obtained on the tumoral antigenic site, at different stages, which is formed by 1) biotinylated mAb, 2) avidin (or streptavidin) and 3) bivalent cytokine modified with the CD13 ligand and biotin. A

number of papers proved that the pre-targeting approach, compared with conventional targeting with immunoconjugates, can actually increase the ratio of active molecule homed at the target to free active molecule, thus reducing the treatment toxicity *(11, 10, 9, 8)*. This approach produced favorable results with biotinylated TNF, which was capable of inducing cytotoxicity *in vitro* and decreasing the tumor cells growth under conditions in which normal TNF was inactive *(14, 26)*. The pre-targeting approach can also be carried out with a two-phase procedure by using a bispecific antibody which at the same time binds the tumoral antigen and the modified cytokine. The use of a bispecific antibody directed against a carcinoembryonic antigen and TNF has recently been described as a means for TNF tumoral pre-targeting *(31)*.

According to a further embodiment, the invention comprises a TNF molecule conjugated to both a CD13 ligand and an antibody, or a fragment thereof (directly or indirectly via a biotin-avidin bridge), on different TNF subunits, where the antibody or its fragments are directed against an antigen expressed on tumor cells or other components of the tumor stroma, e.g. tenascin and fibronectin EDB domain. This results in a further improvement of the tumor homing properties of the modified cytokine and in the slow release of the latter in the tumor microenvironment through trimer-monomer-trimer transitions. As shown in previous works, in fact, the modified subunits of TNF conjugates can dissociate from the targeting complexes and reassociate so as to form unmodified trimeric TNF molecules, which then diffuse in the tumor microenvironment. The release of bioactive TNF has been shown to occur within 24-48 hours after targeting (21).

For use in therapy, the modified cytokines of the invention will be suitably formulated in pharmaceutical preparations for the oral or parenteral

administration. Formulations for the parenteral administration are preferred, and they comprise injectable solutions or suspensions and liquids for infusions. For the preparation of the parenteral forms, an effective amount of the active ingredient will be dissolved or suspended in a sterile carrier, optionally adding excipients such as solubilizers, isotonicity agents, preservatives, stabilizers, emulsifiers or dispersing agents, and it will be subsequently distributed in sealed vials or ampoules.

The preparation of cytokines in form of liposomes can improve the biological activity thereof. It has, in fact, been observed that acylation of the TNF amino groups induces an increase in its hydrophobicity without loss of biological activity in vitro. Furthermore, it has been reported that TNF bound to lipids has unaffected cytotoxicity in vitro, immunomodulating effects and reduced toxicity in vivo *(12, 13)*.

The maximum tolerated dose of bolus TNF in humans is 218-410 $\mu g/m^2$ *(32)* about 10-fold lower than the effective dose in animals. Based on data from murine models it is believed that an at least 10 times higher dose is necessary to achieve anti-tumor effects in humans *(15)*. In the first clinical study on hyperthermic isolated-limb perfusion, high response rates were obtained with the unique dose of 4 mg of TNF in combination with melphalan and interferon γ *(16)*. Other works showed that interferon γ can be omitted and that even lower doses of TNF can be sufficient to induce a therapeutic response *(17, 18)*. As the two cytokines exert synergistic effects on endothelial cells, their combined, selective targeting thereon is likely to result in stronger anti-tumor activity thus allowing to overcome the problems of systemic toxicity usually encountered in cancer therapy with the same cytokines used in combination. Furthermore, it is known that TNF can decrease the barrier function of the endothelial lining vessels, thus increasing their permeability to macromolecules. Taking advantage of the

lower toxicity of treatment with the modified TNF molecules according to the invention, and of their tumor vessels homing properties, an alternative application is their use to increase the permeability of tumor vessels to other compounds, either for therapeutic or diagnostic purposes. For instance the modified TNF can be used to increase the tumor uptake of radiolabelled antibodies or hormones (tumor-imaging compounds) in radioimmunoscintigraphy or radioimmunotherapy of tumors. Alternatively, the uptake of chemotherapeutic drugs, immunotoxins, liposomes carrying drugs or genes, or other anticancer drugs could also be increased, so that their antitumor effects are enhanced.

Accordingly, the cytokines of the invention can be used in combined, separated or sequential preparations, also with other diagnostic or therapeutic substances, in the treatment or in the diagnosis of cancer.

A final aspect of the invention regards the cDNA encoding for the conjugated cytokines herein disclosed, which can be prepared from the cytokines cDNA by addition of a 5'- or 3'-contiguous DNA sequence encoding for the CD13 ligand, preferably for the homing peptides described above. The combined cDNA can be used as such or after insertion in vectors for gene therapy. The preparation and therapeutic applications of suitable vectors is disclosed in *(19)*, which is hereby incorporated by reference.

DESCRIPTION OF THE FIGURES

Figure 1: Effect of TNF and NGR-TNF on the growth of RMA-T lymphomas (a and b) and on the animal weight (c and d).

5 Animals/group were treated with a single dose of TNF or NGR-TNF (i.p.), 10 days after tumor implantation. Tumor area values at day 14 as a function of the dose (b) and the loss of weight after treatment (mean of days 11 and 12) (d), were interpolated from logarithmic curves. The anti-tumor effects induced by 1 μg or 9 μg of NGR-TNF at day 14 were greater than

those induced by comparable amounts of TNF (P = 0.024 and P = 0.032, respectively), while the loss of weight after these treatments was similar. The arrows indicate extrapolated doses of TNF and NGR-TNF that induce comparable effects.

Figure 2: Effect of mAb R3-63 and CNGRC on the anti-tumor activity of NGR-TNF (a) and TNF (b).

MAb R3-63 or CNGRC were mixed with NGR-TNF or TNF and administered to RMA-T tumor bearing animals, 12 days after tumor implantation (n = 5 animals/group). In a separate experiment (c) TNF and NGR-TNF were coadministered with CNGRC or CARAC (a control peptide) to animals bearing 11-day old tumors (n = 5). The anti-tumor effect of 1 μg of NGR-TNF was stronger than that of 9 μg of TNF (P = 0.009, t-test at day 20) and was significantly inhibited by CNGRC (P = 0.035) and by mAb R3-63 (P = 0.011).

Figure 3: Effect of limited tryptic digestion of NGR-TNF and TNF on their mass (a) and anti-tumor activity (b).

Trypsin-agarose was prepared by coupling 1 mg of trypsin to 1 ml of Activated CH Sepharose (Pharmacia-Upjohn), according to the manufacturer's instructions. NGR-TNF and TNF (170 μg each in 300 μl of 0.15 M sodium chloride, 0.05 M sodium phosphate, pH 7.3) were mixed with 15 μl of resin suspension (1:4) or buffer alone and rotated end-over-end at 37°C for the indicated time. The four products were filtered through a 0.22 μm Spin-X device (Costar, Cambridge, MA) and stored at -20°C until use. (a) Electrospray mass spectrometry analysis. The molecular mass values and the corresponding products (N-terminal sequences) are indicated on each peak. The arrows on the sequences indicate the site of cleavage. (b) Effect of 1 or 3 μg of NGR-TNF and TNF, incubated without (upper panels) or with (lower panels) trypsin, on the growth of RMA-T tumors and animal

weight (mean±SE of groups treated with 1 and 3 μg doses). Animals were treated 13 days after tumor implantation (n = 5 animals/group).

Figure 4: SDS-PAGE and anti-tumor activity of human NGR-TNF before and after denaturation/refolding.

SDS-PAGE under non reducing conditions (A) of human TNF (a), NGR-TNF before (b) and after (c) the denaturation/refolding process described in Example V.

Effect of TNF and non-refolded NGR-TNF on the growth of RMA-T lymphomas (B) and on body weight (C). Effect of human TNF (D) and refolded NGR-TNF (consisting of >95% trimers with intra-chain disulfides) (E) on the tumor growth. Animals (15 or 5 mice/group as indicated in each panel) were treated with one i.p. dose of TNF or NGR-TNF, 10 days after tumor implantation.

The following examples further illustrate the invention.

Example I

Preparation of murine TNF and NGR-TNF

Murine recombinant TNF and Cys-Asn-Gly-Arg-Cys-Gly-TNF (NGR-TNF) were produced by cytoplasmic cDNA expression in E.coli. The cDNA coding for murine Met-TNF$_{1-156}$ (20) was prepared by reverse transcriptase-polymerase chain reaction (RT-PCR) on mRNA isolated from lipopolysaccharide-stimulated murine RAW-264.7 monocyte-macrophage cells, using

5'-CTGGATCCTCACAGAGCAATGACTCCAAAG-3' and

5'-TGCCTCACATATGCTCAGATCATCTTCTC-3', as 3' and 5' primers.

The amplified fragment was digested with Nde I and Bam HI (New England Biolabs, Beverley, MA) and cloned in pET-11b (Novagen, Madison, WI), previously digested with the same enzymes (pTNF).

The cDNA coding for Cys-Asn-Gly-Arg-Cys-Gly-TNF$_{1-156}$ was

amplified by PCR on pTNF, using 5'-GCAGATCATATGTGCAACGGCCGTTGCGGCCTCAGATCATCTTCTC-3' as 5' primer, and the above 3' primer. The amplified fragment was digested and cloned in pET-11b as described above and used to transform BL21(DE3) E.coli cells (Novagen). The expression of TNF and NGR-TNF was induced with isopropyl-β-D-thiogalactoside, according to the pET11b manufacturer's instruction. Soluble TNF and NGR-TNF were recovered from two-liter cultures by bacterial sonication in 2 mM ethylenediaminetetracetic acid, 20 mM Tris-HCl, pH 8.0, followed by centrifugation (15000 x g, 20 min, 4°C). Both extracts were mixed with ammonium sulfate (25% of saturation), left for 1 h at 4°C, and further centrifuged, as above. The ammonium sulfate in the supernatants was then brought to 65% of saturation, left at 4°C for 24 h and further centrifuged. Each pellet was dissolved in 200 ml of 1 M ammonium sulfate, 50 mM Tris-HCl, pH 8.0, and purified by hydrophobic interaction chromatography on Phenyl-Sepharose 6 Fast Flow (Pharmacia-Upjohn) (gradient elution, buffer A: 50 mM sodium phosphate, pH 8.0, containing 1 M ammonium sulfate; buffer B: 20% glycerol, 5% methanol, 50 mM sodium phosphate, pH 8.0). Fractions containing TNF immunoreactive material (by western blotting) were pooled, dialyzed against 2 mM ethylenediaminetetracetic acid, 20 mM Tris-HCl, pH 8.0 and further purified by ion exchange chromatography on DEAE-Sepharose Fast Flow (Pharmacia-Upjohn) (gradient elution, buffer A: 20 mM Tris-HCl, pH 8.0; buffer B: 1 M sodium chloride, 20 mM Tris-HCl, pH 8.0). Fractions containing TNF-immunoreactivity were pooled and purified by gel filtration chromatography on Sephacryl-S-300 HR (Pharmacia-Upjohn), pre-equilibrated and eluted with 150 mM sodium chloride, 50 mM sodium phosphate buffer, pH 7.3 (PBS). Fractions corresponding to 40000-50000 Mr products were pooled, aliquoted and

stored frozen at -20°C. All solutions employed in the chromatographic steps were prepared with sterile and endotoxin-free water (Salf, Bergamo, Italy). The final yields were 45 mg of TNF and 34.5 mg NGR-TNF.

The molecular weight of purified TNF and NGR-TNF was measured by electrospray mass spectrometry. The protein content was measured using a commercial protein assay kit (Pierce, Rockford, IL). Endotoxin content of NGR-TNF and TNF was 0.75 units/μg and 1.38 units/μg, respectively, as measured by the quantitative chromogenic Lymulus Amoebocyte Lysate (LAL) test (BioWhittaker).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were carried out using 12.5 or 15% polyacrylamide gels, by standard procedures.

A small amount of TNF and NGR-TNF was further purified by affinity chromatography on soluble p55-TNF receptor (sTNF-R1)-Sepharose as follows: 5 mg of recombinant sTNF-R1 were prepared as described *(22)* and coupled to 2 ml of Activated-CH-Sepharose (Pharmacia), according to the manufacturer's instruction. Two separate columns (one ml each), were washed extensively with sterile and endotoxin-free solutions, loaded with purified TNF or NGR-TNF in PBS and desorbed by gradient elution (1 h, buffer A: PBS; buffer B: 0.5 M sodium chloride, 0.2 M glycine-HCl). The TNF-antigen containing fractions were neutralized and dialyzed against sterile physiological solution. Endotoxin-free human serum albumin was added before dialysis (0.5 mg/ml) to prevent protein adsorption on membranes. The TNF content in each fraction was measured by ELISA and cytolytic assay.

Non reducing SDS-PAGE of TNF showed a single band of 17-18 kDa, as expected for monomeric TNF (not shown). At variance, non reducing SDS-PAGE and western blot analysis of NGR-TNF showed

different immunoreactive forms of 18, 36 and 50 kDa likely corresponding to monomers, dimers and trimers. Under reducing conditions most of the 50 and 36 kDa bands were converted into the 18 kDa form, pointing to the presence of NGR-TNF molecules with interchain disulfide bridges. The 18 kDa band accounted to about 2/3 of the total material, whereas the 36 kDa accounted for most of the remaining part. These electrophoretic patterns suggest that NGR-TNF was a mixture of trimers made up by three monomeric subunits with correct intra-chain disulfides (at least 50%) and the remaining part mostly by trimers with one or more interchain disulfides. The 36 kDa band still observed by reducing SDS-PAGE suggests that NGR-TNF contained also an irreversible denatured dimer (about 10% of total).

The molecular mass of TNF and NGR-TNF monomers were 17386.1±2.0 Da and 17843.7±2.5 Da, respectively, by electrospray mass spectrometry. These values correspond very well to the mass expected for Met-TNF_{1-156} (17386.7 Da) and for CNGRCG-TNF_{1-156} (17844.2 Da).

Example II

In vitro cytotoxic activity of murine TNF and NGR-TNF

The bioactivity of TNF and NGR-TNF was estimated by standard cytolytic assay based on L-M mouse fibroblasts (ATCC CCL1.2) as described *(23)*. The cytolytic activity of TNF and NGR-TNF on RMA-T cells was tested in the presence of 30 ng/ml actinomycin D. Each sample was analyzed in duplicate, at three different dilutions. The results are expressed as mean ± SD of two-three independent assays.

The in vitro cytotoxic activity of TNF and NGR-TNF was (1.2±0.14) x 10^8 units/mg and (1.8±0.7) x 10^8 units/mg, respectively, by standard cytolytic assay with L-M cells. These results indicate that the CNGRCG moieties in the NGR-TNF molecule does not prevent folding, oligomerization and binding to TNF receptors.

In a previous study we showed that RMA-T cells can be killed by TNF in the presence of 30 ng/ml actinomycin D, whereas in the absence of transcription inhibitors these cells are resistant to TNF, even after several days of incubation. The in vitro cytotoxic activity of NGR-TNF on RMA-T cells in the presence of actinomycin D was $(1.4\pm0.8) \times 10^8$ units/mg, as measured using TNF ($(1.2\pm0.14) \times 10^8$ units/mg) as a standard. Thus, the cytotoxic activities of NGR-TNF and TNF were similar both on L-M and RMA-T cells.

Example III

Characterization of the therapeutic and toxic activity of murine TNF and NGR-TNF

The Rauscher virus-induced RMA lymphoma of C57BL/6 origin, were maintained in vitro in RPMI 1640, 5% foetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM glutamine and 50 µM 2-mercaptoethanol. RMA-T was derived from the RMA cell line by transfection with a construct encoding the Thy 1.1 allele and cultured as described *(14)*.

B16F1 melanoma cells were cultured in RPMI 1640, 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM glutamine, 1% MEM non essential amino acid (BioWhittaker Europe, Verviers, Belgium).

In vivo studies on animal models were approved by the Ethical Committee of the San Raffaele H Scientific Institute and performed according to the prescribed guidelines. C57BL/6 (Charles River Laboratories, Calco, Italy) (16-18 g) were challenged with 5×10^4 RMA-T or B16F1 living cells, respectively, s.c. in the left flank. Ten-twelve days after tumor implantation, mice were treated, i.p., with 250 µl TNF or NGR-TNF solutions, diluted with endotoxin-free 0.9% sodium chloride.

Preliminary experiments showed that the anti-tumor activity was not changed by the addition of human serum albumin to TNF and NGR-TNF solutions, as a carrier. Each experiment was carried out with 5 mice per group. The tumor growth was monitored daily by measuring the tumor size with calipers. The tumor area was estimated by calculating $r_1 \times r_2$, whereas tumor volume was estimated by calculating $r_1 \times r_2 \times r_3 \times 4/3$, where r_1 and r_2 are the longitudinal and lateral radii, and r_3 is the thickness of tumors protruding from the surface of normal skin. Animals were killed before the tumor reached 1.0-1.3 cm diameter. Tumor sizes are shown as mean$\pm$SE (5-10 animals per group as indicated in the figure legends) and compared by t-test.

The anti-tumor activity and toxicity of NGR-TNF were compared to those of TNF using the RMA-T lymphoma and the B16F1 melanoma models in C57BL6 mice. Since the RMA-T model has been previously characterized and used to study the anti-tumor activity of TNF with different targeting protocols *(26)* we decided to use this model also in this study.

Murine TNF administered to animals bearing established s.c. RMA-T tumors, causes 24 h later a reduction in the tumor mass and haemorragic necrosis in the central part of the tumor, followed by a significant growth delay for few days *(26)*. A single treatment with TNF does not induce complete regression of this tumor, even at doses close to the LD50, as living cells remaining around the necrotic area restart to grow few days after treatment.

In a first set of experiments we investigated the effect of various doses (i.p.) of TNF or NGR-TNF on animal survival. To avoid excessive suffering, the animals were killed when the tumor diameter was greater than 1-1.3 cm. The lethality of TNF and NGR-TNF, 3 days after treatment, was

similar (LD50, 60 μg and 45 μg, respectively) whereas their anti-tumor activity was markedly different (Table 1).

Table 1. Survival of mice with RMA-T lymphoma treated with murine TNF or NGR-TNF

Treatment	Animals (n)	Dose (μg)	Survival (%)[a] after treatment							
			Day 3	Day 7	Day 14	Day 21	Day 30	Day 38 (2nd ch)[b]	Day 62 (3° ch.)[b]	Day 92
None	18	0	100	0						
TNF	4	1	100	20	0					
	9	3	100	55	0					
	9	9	100	55	22	11	0			
	14	27	100	57	14	7	0			
	9	54	55	55	0					
	9	108	0							
NGR-TNF	10	1	100	70	10	10	10	0		
	10	3	100	80	20	20	20	0		
	9	9	100	88	55	22	11	11	11	
	13	27	100	85	30	23	15	15	15	11
	9	54	33	33	0					15
	9	108	0							

a) Animals with tumor were treated with TNF or NGR-TNF (i.p.) 10 days after tumor implant. Animals were killed when the tumor diameter exceeded 1-1.3 cm.

b) Surviving animals were re-challenged with 50,000 RMA-T cells (second challenge) or 50,000 RMA (third) at the indicated time. Tumorigenicity of injected cells was monitored at each time with the 5 normal animals. All control animals developed a tumor within 10 days (data not shown).

For instance, 1 or 3 μg of NGR-TNF delayed tumor growth more efficiently then 27 μg of TNF, indicating that NGR-TNF was at least one order of magnitude more active. Interestingly, some animals were cured with doses of NGR-TNF lower than the LD50, whereas no animals at all were cured with TNF. Cured animals rejected further challenges with tumorigenic doses of either RMA-T or wild-type RMA cells, suggesting that a single treatment with NGR-TNF was able to induce protective immunity. It is noteworthy that increasing the dose of TNF or NGR-TNF above 9-27 μg markedly increased the toxicity and poorly or not the therapeutic activity.

The loss of weight consequent to TNF treatment is a well known sign of systemic toxicity (*26*). Thus, to further compare the efficacy/toxicity ratio of TNF and NGR-TNF we monitored the tumor growth and the animal weight after treatment. The effect of 1 μg of NGR-TNF on the tumor growth was similar or higher than that of 9 μg of TNF (Fig. 1a), while the loss of weight one-two days after treatment was comparable to that of 1 μg of TNF (Fig. 1c). When we interpolated the data with a logarithmic curve in a dose-response plot we found that the therapeutic effect of 9 μg of TNF at day 14 can be obtained with as little as 0.6 μg of NGR-TNF (Fig.1b). In contrast, 8.5 μg were necessary to induce a comparable toxic effect (Fig. 1d). Thus, the calculated efficacy/toxicity ratio of NGR-TNF under these conditions is 14 times greater than that of TNF.

Similar results were obtained with the B16F1 melanoma model. Treatment with 1 μg of NGR-TNF at day 11 and day 17, induced an anti-tumor response at day 19 greater than that obtained with 4 μg of TNF and similar to that obtained with 12 μg of TNF (data not shown). In contrast, the loss of weight caused by 1 μg of NGR-TNF was markedly lower than that caused by 4 and 12 μg of TNF. Treatment with 12 μg of NGR-TNF caused

an even stronger anti-tumor effect, while the toxic effect was similar to that of 12 μg of TNF.

When a third injection was done on day 19 some animal deaths occurred 1-2 days later in all groups (2 out of 5 in the group treated with saline and 12 μg of NGR-TNF and 1 out of 5 in the remaining groups). Of note, one animal treated with 12 μg of NGR-TNF completely rejected the tumor. When this animal was challenged with a second tumorigenic dose of B16F1 cells, a palpable tumor developed after 18 days, while control animals developed a tumor within 6-7 days.

These results, altogether, suggest that the efficacy of NGR-TNF in inhibiting the tumor growth is 10-15 times greater than that of TNF whereas the toxicity is similar. Moreover, NGR-TNF can induce protective immune responses more efficiently than TNF.

Example IV

Mechanism of action of NGR-TNF

Anti-mouse CD13 mAb R3-63 purified from ascitic fluids by protein-G Sepharose chromatography (Pharmacia-Upjohn, Uppsala, Sweden), and dialyzed against 0.9% sodium chloride.

Rabbit polyclonal antiserum was purchased from Primm srl (Milan, Italy) and purified by affinity chromatography on protein-A-Sepharose (Pharmacia-Upjohn). CNGRC and CARAC peptides were prepared as described previously *(28)*.

To provide evidence that the improved activity of NGR-TNF is dependent on tumor targeting via the NGR moiety we have investigated whether the in vivo activity of NGR-TNF can be partially competed by CNGRC. To this end we have administered NGR-TNF (1 μg) to RMA-T tumor bearing mice, with or without a molar excess of CNGRC. In parallel, other animals were treated with TNF (3 or 9 μg), again with or without

CNGRC. As expected, CNGRC decreased significantly the anti-tumor activity of NGR-TNF (Fig. 2a) but not that of TNF (Fig. 2b). At variance, a control peptide (CARAC) was unable to cause significant decrease of NGR-TNF activity (Fig. 2c). These results suggest that CNGRC competes for the binding of NGR-TNF to a CNGRC receptor, and support the hypothesis of a targeting mechanism for the improved activity. Of note, CNGRC was unable to decrease the in vitro cytotoxic activity of NGR-TNF (data not shown).

Since it has been recently reported that aminopeptidase N (CD13) is a receptor for CNGRC peptides, we then investigated the contribute of this receptor in the targeting mechanism of NGR-TNF. To this end, we studied the effect of an anti-CD13 mAb (R3-63) on the anti-tumor activity of NGR-TNF and TNF. MAb R3-63 significantly inhibited the anti-tumor activity of NGR-TNF (Fig. 2a) but not that of TNF (Fig. 2b) indicating that CD13 is indeed critically involved in the anti-tumor activity of NGR-TNF. No expression of CD13 on RMA-T cell surface was observed by FACS analysis of cultured cells with mAb R3-63 (not shown), suggesting that other cells were recognized by the antibody in vivo.

Although these data indicate that CD13 is an important receptor for NGR-TNF, we cannot entirely exclude that binding to other not yet identified NGR receptors also contribute, albeit to a lower extent, to the targeting mechanism.

Preliminary experiments of partial proteolysis showed that the Arg-Ser bond in the N-terminal segment of TNF (residues 2-3) is very sensitive to trypsin, whereas the rest of the molecule is much more resistant. Thus, to provide further evidence that the improved activity of NGR-TNF is related to its NGR moiety, we tried to cleave out the NGR domain from the N-terminal region of the mutein by partial digestion with immobilized trypsin. This treatment converted both NGR-TNF and TNF into a molecule

corresponding to the TNF3-156 fragment (expected mass 16986.2 Da; see Fig. 3a for measured mass and expected sequences).

While digestion did not decrease the in vitro cytolytic activity of NGR-TNF on L-M cells ($2.3\pm1.4)x10^8$ U/mg) its in vivo anti-tumor activity was decreased to the level of TNF (Fig. 3b). Of note, the toxicity of NGR-TNF and TNF were similar both before and after digestion, as judged from animal weight loss one day after treatment (Fig. 3b, right panel), suggesting that the NGR-dependent targeting mechanism does not alters the toxicity.

Example V

Preparation and characterization of human TNF and NGR-TNF

Human recombinant TNF and NGR-TNF (consisting of human TNF1-157 fused with the C terminus of CNGRCG) were prepared by recombinant DNA technology and purified essentially as described for murine TNF and NGR-TNF. The cDNA coding for human NGR-TNF was prepared by PCR on plasmid pET11b/hTNF containing the hTNF coding sequence *(33)*, using the following primers:

- NGR-hTNF/1 (sense): 5'A TAT CAT ATG TGC AAC GGC CGT TGC GGC GTC AGA TCA TCdT TCT CG 3'.
- NGR-hTNF/2 (antisense): 5' TCA GGA TCC TCA CAG GGC AAT GAT CCC AAA GTA GAC 3'.

The amplified fragment was digested and cloned in pET-11b (Nde I/BamH I) and used to transform BL21(DE3) E.coli cells (Novagen). The expression of NGR-hTNF was induced with isopropyl-β-D-thiogalactoside, according to the pET11b manufacturer's instruction. Soluble NGR-TNF was recovered from two-liter cultures by bacterial sonication in 2 mM ethylenediaminetetracetic acid, 20 mM Tris-HCl, pH 8.0, followed by centrifugation (15000 x g, 20 min, 4°C).

The extract was mixed with ammonium sulfate (35% of saturation), left for 1 h at 4°C, and further centrifuged, as above. The ammonium sulfate

in the supernatants was then brought to 65% of saturation, left at 4°C for 24 h and further centrifuged. Each pellet was dissolved in 1 M ammonium sulfate, 50 mM Tris-HCl, pH 8.0, and purified by hydrophobic interaction chromatography on Phenyl-Sepharose 6 Fast Flow (Pharmacia-Upjohn) (gradient elution, buffer A: 100 mM sodium phosphate, pH 8.0, containing 1 M ammonium sulfate; buffer B: 70% ethylen glycol, 5% methanol, 100 mM sodium phosphate, pH 8.0). Fractions containing hTNF immunoreactive material (by ELISA) were pooled, dialyzed against 20 mM Tris-HCl, pH 8.0 and further purified by ion exchange chromatography on DEAE-Sepharose Fast Flow (Pharmacia-Upjohn) (gradient elution, buffer A: 20 mM Tris-HCl, pH 8.0; buffer B: 1 M sodium chloride, 20 mM Tris-HCl, pH 8.0). All solutions employed in the chromatographic steps were prepared with sterile and endotoxin-free water (Salf, Bergamo, Italy).

At this point about 30 mg of TNF and 32 mg NGR-TNF was recovered from two-liters cultures. Non reducing SDS-PAGE showed bands corresponding to monomers, dimers and trimers suggesting that also human NGR-TNF was a mixture of trimers with correct intra-chain disulfides and trimers with one or more interchain disulfide bridges (Fig. 4A, lane b), as observed with murine NGR-TNF.

Trimers with correct intrachain disulfide bridges were isolated from this mixture by a four-step denaturation-refolding process as follows: purified human NGR-TNF was denatured with 7 M urea and gelfiltered through an HR Sephacryl S-300 column (1025 ml) (Pharmacia) pre-equilibrated with 7 M urea, 100 mM Tris-HCl, pH 8.0. Fractions corresponding to monomeric TNF were pooled, ultrafiltered through an YM MWCO 10 kDa membrane (Amicon) and refolded by dialysis against 33 volumes of 2.33 M urea, 100 mM Tris-HCl, pH 8 at 4°C (140 min) followed by 1.55 M urea, 100 mM Tris-HCl, pH 8 (140 min) and 1 M urea, 100 mM

Tris-HCl, pH 8 (140 min). Finally the product was dialyzed against 80 volumes of 100 mM Tris-HCl (16 h), centrifuged at 13000 x g (30 min), filtered through a SFCA 0.45 μm membrane (Nalgene) and gelfiltered through an HR Sephacryl S-300 column (1020 ml) pre-equilibrated with 0.15 M sodium chloride, 0.05 M sodium phosphate (PBS). About 23 mg of refolded protein was recovered.

The final product was mostly monomeric after non reducing SDS-PAGE (Fig. 4A, lane c), had an hydrodynamic volume similar to that of trimeric human TNF by analytical gel-filtration HPLC on a Superdex 75 HR column (not shown), and had a molecular mass of 17937.8 + 1.8 Da (expected for CNGRCG-TNF1-157, 17939.4 Da) by electrospray mass spectrometry. The in vitro cytolytic activities of non-refolded and refolded NGR-TNF on mouse L-M cells were (6.11 x 107) + 4.9 and (5.09 x 107) + 0.3 units/mg respectively, whereas that of purified human TNF was (5.45 x 107) + 3.1 units/mg. These results suggest that the denaturation-refolding process did not affect the interaction of human NGR-TNF with the murine p55 receptor.

The in vivo anti-tumor activity of 1 μg of human NGR-TNF (non refolded) was greater than that of 10 μg of TNF (Fig. 4B) whereas the toxicity, as judged by animal weight loss, was significantly lower (Fig. 4C). After refolding 0.3 μg of NGR-TNF was sufficient to induce an anti-tumor effect stronger than that achieved with 10 μg of TNF (Fig. 4D, 4E).

These results indicate that the anti-tumor activity of human NGR-TNF is greater than that of human TNF.

Furthermore, we have observed that refolded human and mouse NGR-TNF can induce significant anti-tumor effects on RMA-T-bearing mice even at very low doses (1-10 ng/mouse) with no evidence of toxic effects, while TNF was unable to induce significant effects at these doses (not shown).

Example VI

Preparation and characterization of mouse NGR-IFNγ

Recombinant murine interferon (IFN)γ fused with CNGRCG (NGR-IFNγ) was prepared by recombinant DNA technology, essentially as described for NGR-TNF. The CNGRC domain was fused with the C terminus of IFNγ. Moreover the cysteine in position 134 was replaced with a serine; a methionine was introduced in position –1 for expression in E.coli cells. The PCR primers used for the production of the NGR-IFNγ cDNA were: 5'-A TAT CTA CAT ATG CAC GGC ACA GTC ATT GAA AGC C (sense) and 5'-TC GGA TCC TCA GCA ACG GCC GTT GCA GCC GGA GCG ACT CCT TTT CCG CTT CCT GAG GC. The cDNA was cloned cloned in pET-11b (Nde I/BamH I) and used to transform BL21(DE3) E.coli cells (Novagen). Protein expression was induced with isopropyl-β-D-thiogalactoside, according to the pET11b manufacturer's instruction. The product was purified from E.coli extracts by immunoaffinity chromatography using an anti-mouse IFN γ mAb (AN18) immobilized on agarose, according to standard techniques. Reducing and non reducing SDS-PAGE of the final product showed a single band of 16 kDa. Electrospray mass spectrometry showed a molecular weight of 16223 + 3.6 Da (expected, 1625.5 Da) corresponding to murine Met-IFNγ1-134(C134S)CNGRC (NGR-IFNγ).

The capability of NGR-IFNγ and NGR-TNF to compete the binding of an anti-CD13 antibody to tumor associated vessels was investigated by using an immunohistochemical approach.

Fresh surgical specimens of human renal cell carcinoma were obtained from the Histopathology Department of the San Raffaele H Scientific Institute. Sections (5-6 μm thick) of Bouin-fixed (4-6 h) paraffin-embedded specimens were prepared and adsorbed on polylysine-coated slides. CD13 antigen were detected using the avidin-biotin complex method as follows:

tissue sections were rehydrated using xylenes and graded alcohol series, according to standard procedures. Tissue sections were placed in a vessel containing 1 mM EDTA and boiled for 7 min using a micro-wave oven (1000 W). The vessel was then refilled with 1 mM EDTA and boiled again for 5 min. The tissue sections were left to cool and incubated in PBS containing 0.3% hydrogen peroxide for 15 min, to quench endogenous peroxidase. The samples were then and rinsed with PBS and incubated with 100-200 µl of PBS-BSA (1 h at room temperature) followed by the mAb WM15 (anti-hCD13), alone or mixed with various competitor agents (see Table 2) in PBS-BSA (overnight at 4°C). The slides were then washed 3 times (3 min each) with PBS and incubated with PBS-BSA containing 2% normal horse serum (PBS-BSA-NHS) (Vector Laboratories, Burlingame, CA) for 5 min. The solution was then replaced with 3 µg/ml biotinylated horse anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA) in PBS-BSA-NHS and further incubated for 1 h at room temperature. The slides were washed again and incubated for 30 min with Vectastain Elite Reagent (Vector Laboratories, Burlingame, CA) diluted 1:100 in PBS. A tablet of 3,3'-diamino-benzidine-tetrahydrocloride (Merck, Darmstadt, Germany) was then dissolved in 10 ml of deionized water containing 0.03% hydrogen peroxide, filtered through a 0.2 µm membrane and overlaid on tissue sections for 5-10 min. The slides were washed as above and counterstained with Harris' hematoxylin. The tumor associated vessels were identified by staining serial sections of the tissue with an anti-CD31 mAb (mAb JC/70A, anti-human CD31, IgG1, from DAKO, Copenhagen, Denmark).

The results are summarized in Table 2. As shown , the binding of WM15 to tumor associated vessels was inhibited by an excess of NGR-TNF, NGR-IFNγ and CNGRC, but not by other control reagents lacking the NGR

motif. This suggests that the NGR binding site on CD13 sterically overlaps with the WM15 epitope. In contrast, NGR-TNF was unable to compete the binding of 13C03 to epithelial cells.

We conclude that the NGR moiety of NGR-IFNγ and NGR-TNF and can interact with a CD13 form recognized by mAb WM15 on tumor associated vessels. Moreover, these results indicate that the CNGRC motif is functional either when linked to the N-terminus or the C-terminus of a cytokine.

Table 2. Binding of WM15 to renal cell cancer sections in the presence of various competitors

Competitor	Binding of WM15 to tumor associated vessels
None	+
NGR-TNF (25 μg/ml)	-
NGR-IFNγ (50 μg/ml)	-
CNGRC (100 μg/ml)	-
TNF (25 μg/ml)	+
Human serum albumin (25 μg/ml)	+
Synthetic CgA(60-68) (100 μg/ml)	+

[a] The competitor, in PBS containing 2% BSA, was added in the blocking step and mixed with the primary antibody.

[b]mAb WM15 (anti-human CD13, IgG1) was from Pharmingen (San Diego, CA);

the synthetic peptide CgA(60-68) corresponds to the chromogranin A

fragment 60-68.

Example VII

Targeted delivery of biotinylated NGR-TNF to tumors using anti-tumor antibodies and avidin (pre-targeting)

The following example illustrates the possibility of "dual" targeting of TNF, based on the combination of a tumor homing antibody and the peptide CNGRC.

A biotin-NGR-TNF conjugate was prepared by mixing NGR-TNF with D-biotinyl-6-aminocaproic acid N-hydroxysuccinimide ester (Societá Prodotti Antibiotici S.p.A, Milan, Italy), in 1 M sodium-carbonate buffer, pH 6.8 (3 h at room temperature) (21). The reaction was blocked with 1 M Tris-HCl, pH 7.5.

The conjugate was characterized by mass spectrometry and found to contain 1 biotin/trimer (on average). C57BL/6 (Charles River Laboratories, Calco, Italy) were then challenged with 5 x 10^4 RMA-T living cells, s.c. in the left flank. When the tumor area reached 40 mm^2, mice were treated by sequential injections of biotinylated antibody, avidins and biotin-TNF according to a "three-day" protocol as described previously (26). We injected: 40 μg biotin-mAb19E12 (i.p., step I), 60 μg avidin and 60 μg streptavidin after 18 and 19 h, respectively (i.p., step II), 3 μg of biotin-NGR-TNF, 24 h later (i.p, step III). Each compound was diluted with a sterile 0.9 % sodium chloride solution. In control experiment, avidin and streptavidin were omitted. Each experiment was carried out with 5 mice/group. The tumor growth was monitored daily by measuring the tumor size with calipers. The tumor areas before and 10 days after treatment were 39 ± 4 mm^2 and 8 ± 5 mm^2, respectively, in the group treated with mAb 19E12-biotin/avidin/streptavidin/biotin-NGR-TNF (5 animals, mean±SE) . In the control group (treated with mAb 19E12-biotin /biotin-NGR-TNF

alone) the tumor areas before and 10 days after treatment were 40 ± 4 mm^2 and 20 ± 6 mm^2 respectively, indicating that pre-targeting with tumor homing antibody and avidin has increased the activity of NGR-TNF.

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CLAIMS

1. A conjugation product between a cytokine selected from TNF or IFNγ and a ligand of the CD13 receptor.

2. A conjugation product as claimed in claim 1, wherein said cytokine is TNFα or TNF β.

3. A conjugation product as claimed in claims 1-2, wherein the ligand of the CD13 receptor is selected from the group consisting of antibodies or active fragments thereof, peptides or peptido-mymetics.

4. A conjugation product as claimed in claim 3, wherein said ligand is a peptide containing the NGR motif.

5. A conjugation product as claimed in claim 4, wherein said peptide is selected from the group consisting of CNGRCVSGCAGRC, NGRAHA, GNGRG, cycloCVLNGRMEC, linear or cyclic CNGRC.

6. A conjugation product as claimed in any one of the preceding claims, wherein the cytokine is derivatized with polyethylene glycol or an acyl residue.

7. A conjugation product as claimed in any one of the preceding claims, wherein the cytokine is further conjugated with an antibody, or a fragment thereof, directed to a tumoral antigen, a tumoral angiogenic marker or a component of the extracellular matrix, or with biotin.

8. A conjugation product according to claim 7, wherein the cytokine is TNF and is conjugated to both a CD13 ligand and, alternatively, an antibody, a fragment thereof, or biotin, on different subunits.

9. A cDNA encoding for a cytokine selected from TNF and IFN bearing a 5' or 3' contiguous sequence encoding a CD13 ligand.

10. A cDNA according to claim 9, wherein said CD13 ligand is a peptide according to claim 5.

11. A vector for gene therapy containing the cDNA of claims 9-10.

12. Pharmaceutical composition comprising an effective amount of a conjugation product as claimed in claims 1-8, together with pharmaceutically acceptable carriers and excipients.

13. A composition as claimed in claim 12, in the form of an injectable solution or suspension or a liquid for infusions.

14. A composition as claimed in claims 12-13, in the form of liposomes.

15. The use of a conjugation product as claimed in claims 1-8 or a cDNA according to claims 9-10, for the preparation of medicaments or diagnostics for the therapy of cancer.

16. The use of a conjugation product according to claim 15, in combination with other antitumor agents or diagnostic tumor-imaging compounds.

FIGURE 1

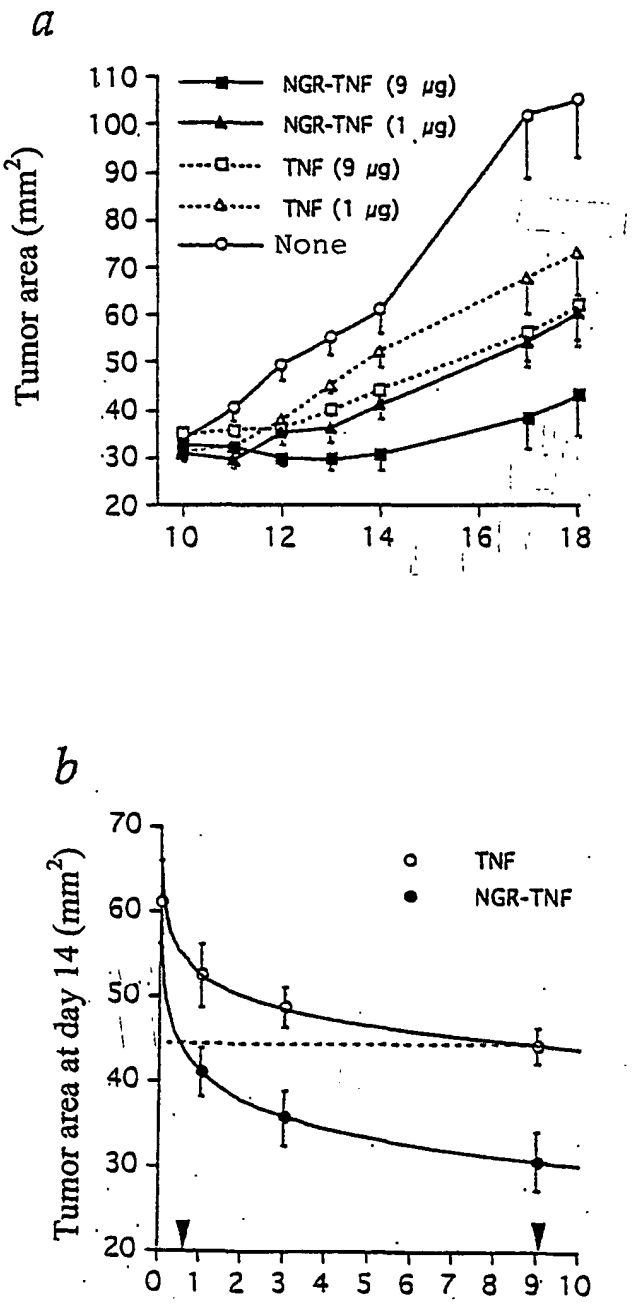
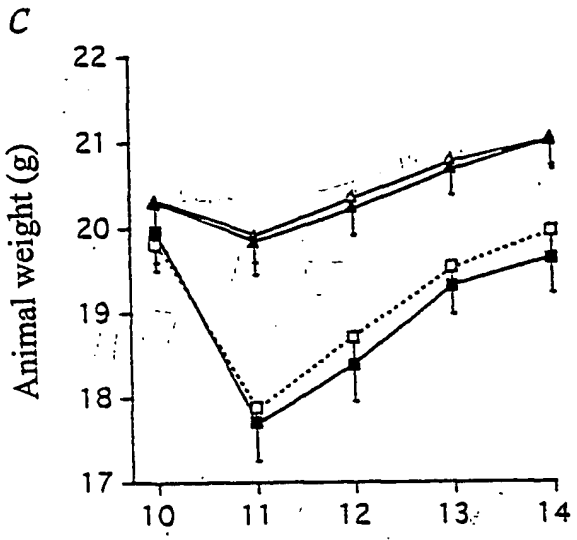

a
Tumor area (mm²)
NGR-TNF (9 μg)
NGR-TNF (1 μg)
TNF (9 μg)
TNF (1 μg)
None
110
100
90
80
70
60
50
40
30
20
10
12
14
16
18
b
Tumor area at day 14 (mm²)
TNF
NGR-TNF
70
60
50
40
30
20
0
1
2
3
4
5
6
7
8
9
10

FIGURE 1


c
Animal weight (g)
22
21
20
19
18
17
10
11
12
13
14

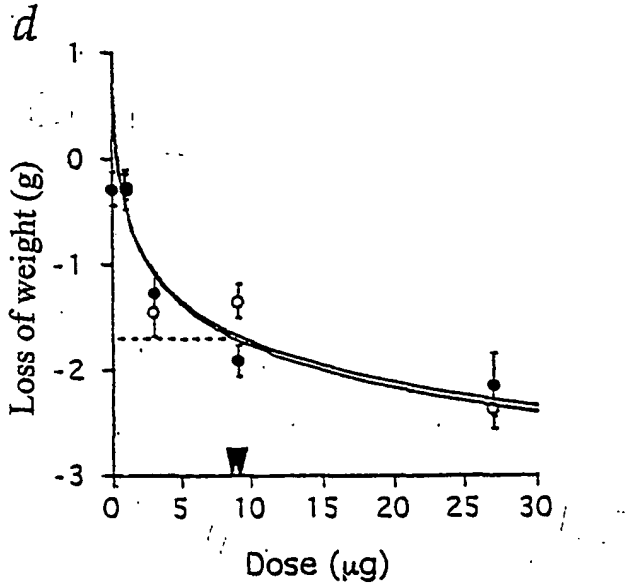

d
Loss of weight (g)
1
0
-1
-2
-3
0
5
10
15
20
25
30
Dose (µg)

FIGURE 2

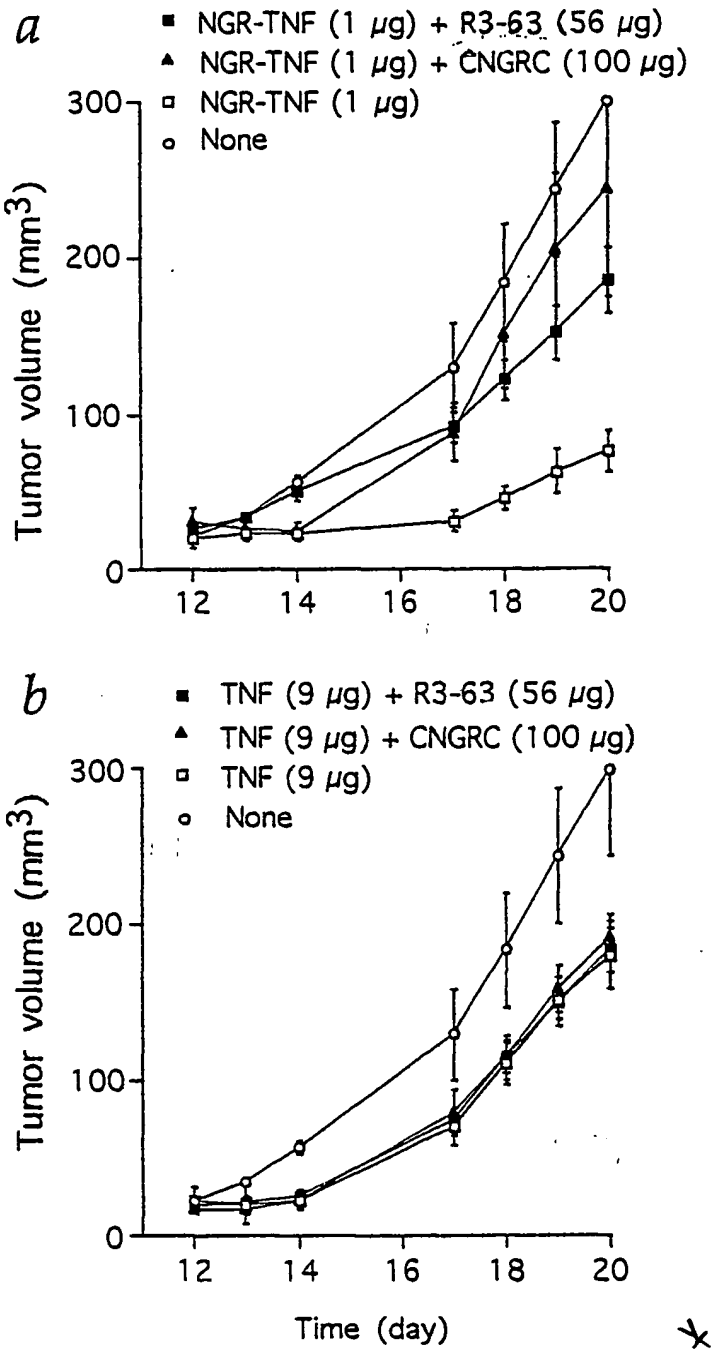

a
NGR-TNF (1 μg) + R3-63 (56 μg)
NGR-TNF (1 μg) + CNGRC (100 μg)
NGR-TNF (1 μg)
None
Tumor volume (mm3)
300
200
100
0
12
14
16
18
20
b
TNF (9 μg) + R3-63 (56 μg)
TNF (9 μg) + CNGRC (100 μg)
TNF (9 μg)
None
Tumor volume (mm3)
300
200
100
0
12
14
16
18
20
Time (day)

FIGURE 2

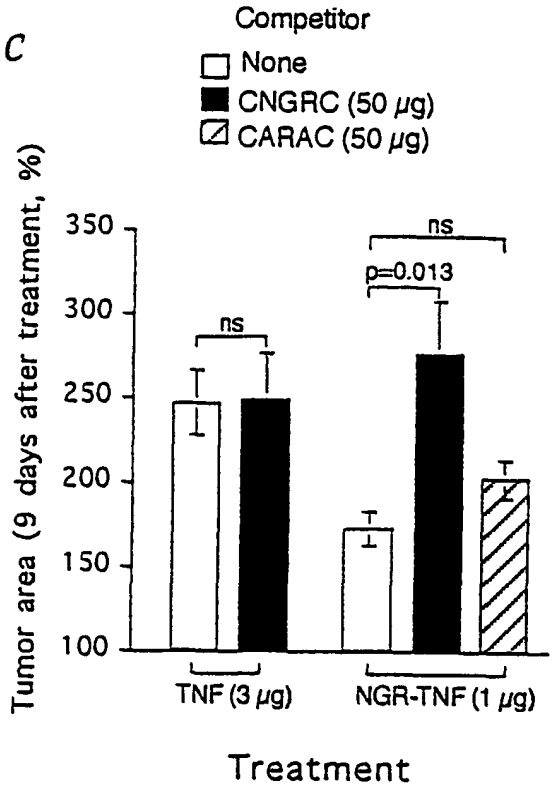

C
Competitor
None
CNGRC (50 μg)
CARAC (50 μg)
Tumor area (9 days after treatment, %)
350
300
250
200
150
100
ns
p=0.013
ns
TNF (3 μg)
NGR-TNF (1 μg)
Treatment

FIGURE 3

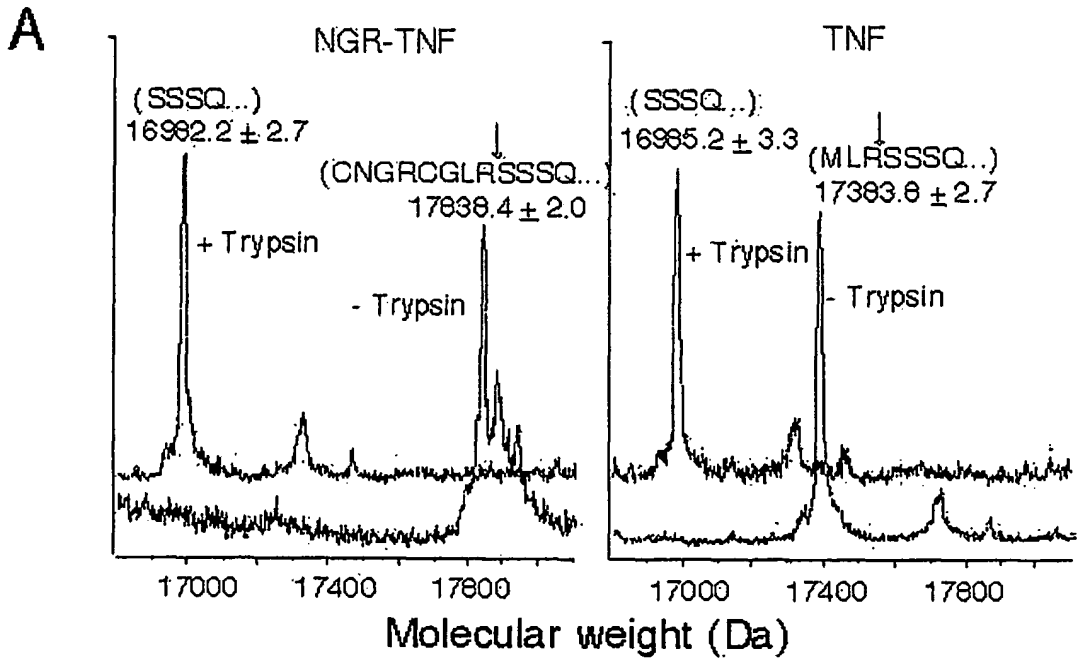

A
NGR-TNF
(SSSQ...)
16982.2 ± 2.7
(CNGRCGLRSSSQ...)
17638.4 ± 2.0
+ Trypsin
- Trypsin
TNF
(SSSQ...)
16985.2 ± 3.3
(MLRSSSQ...)
17383.6 ± 2.7
+ Trypsin
- Trypsin
17000
17400
17800
17000
17400
17800
Molecular weight (Da)

FIGURE 3

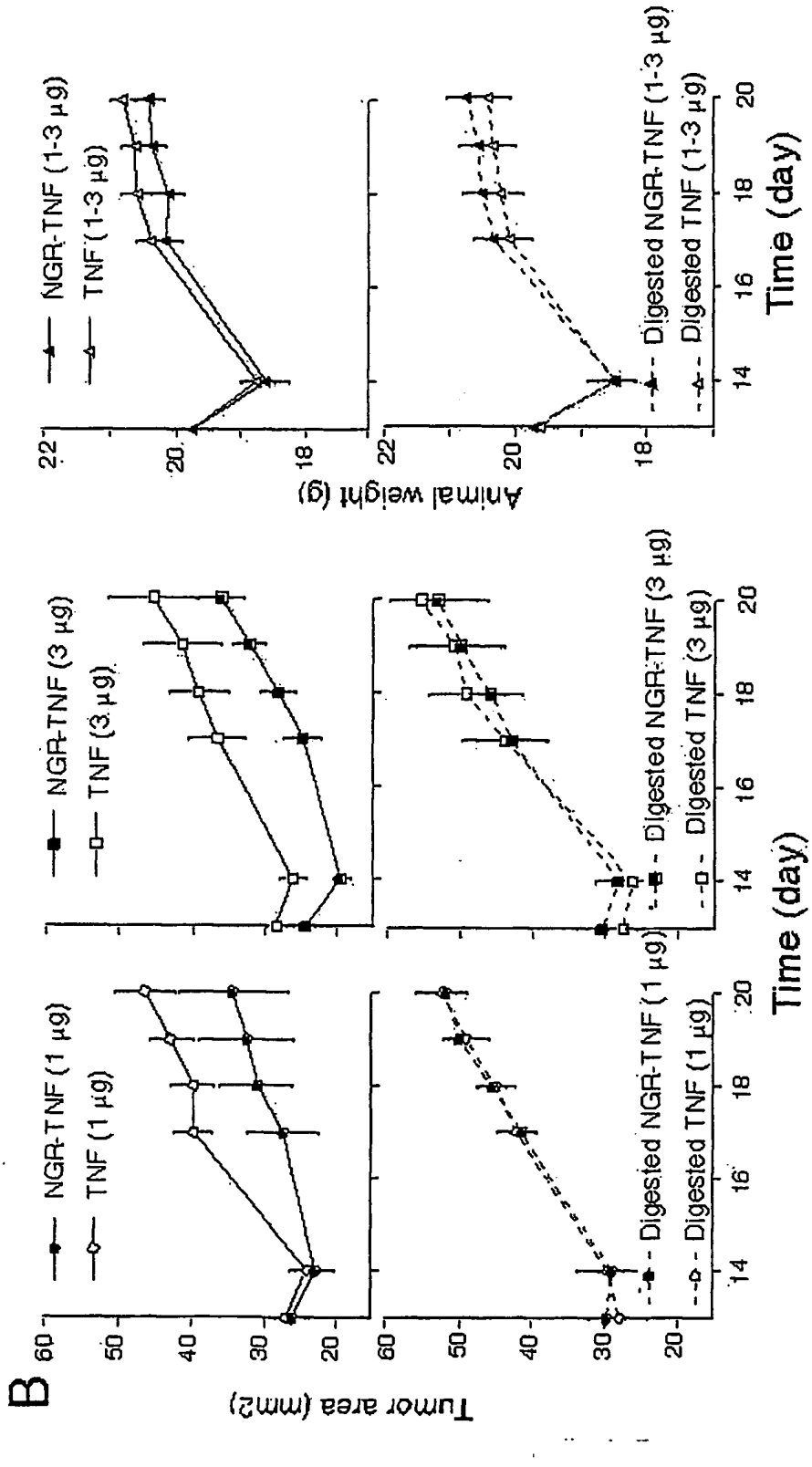

B
Tumor area (mm2)
NGR-TNF (1 µg)
TNF (1 µg)
NGR-TNF (3 µg)
TNF (3 µg)
Digested NGR-TNF (1 µg)
Digested TNF (1 µg)
Digested NGR-TNF (3 µg)
Digested TNF (3 µg)
Time (day)
Animal weight (g)
NGR-TNF (1-3 µg)
TNF (1-3 µg)
Digested NGR-TNF (1-3 µg)
Digested TNF (1-3 µg)
Time (day)

FIGURE 4

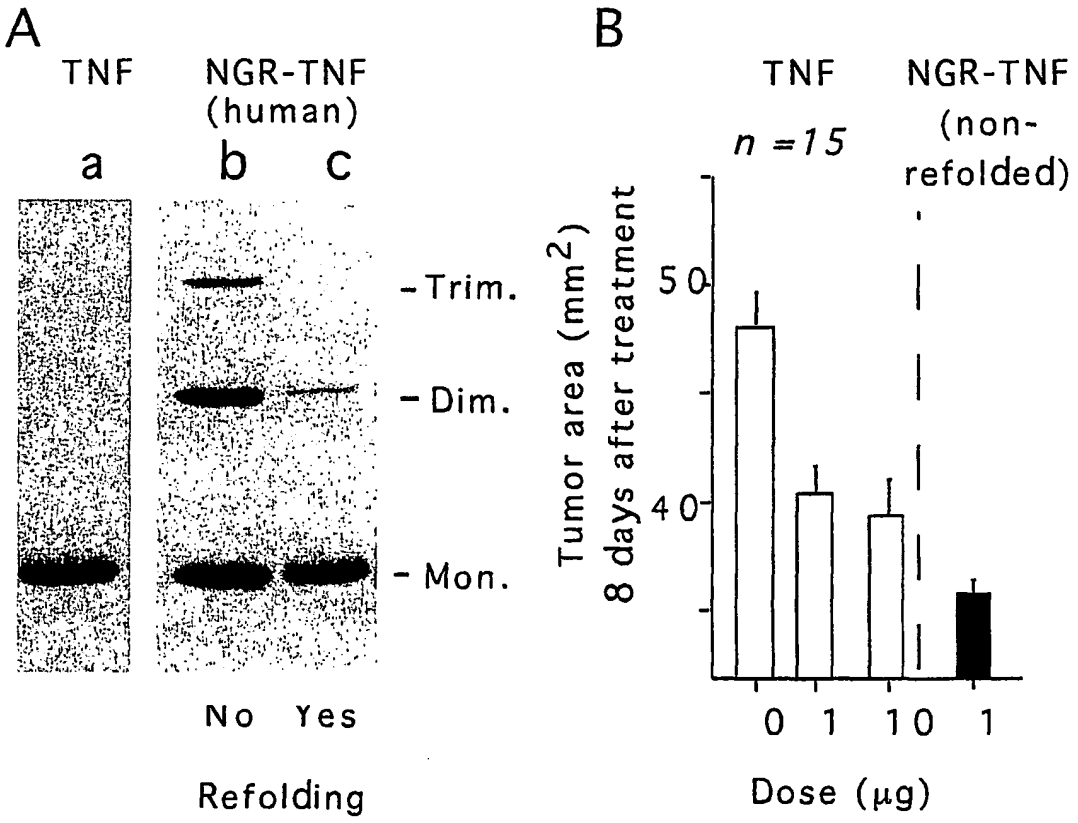

A
TNF
NGR-TNF (human)
a
b
c
- Trim.
- Dim.
- Mon.
No Yes
Refolding
B
TNF
n =15
NGR-TNF (non-refolded)
Tumor area (mm2) 8 days after treatment
50
40
0 1 10 1
Dose (μg)

FIGURE 4

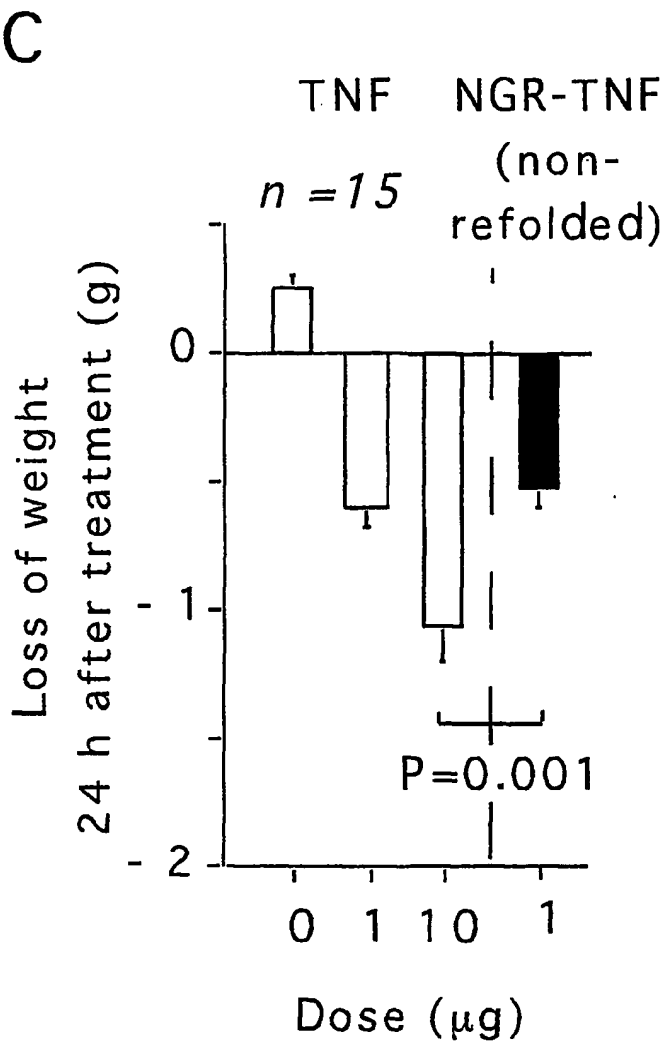
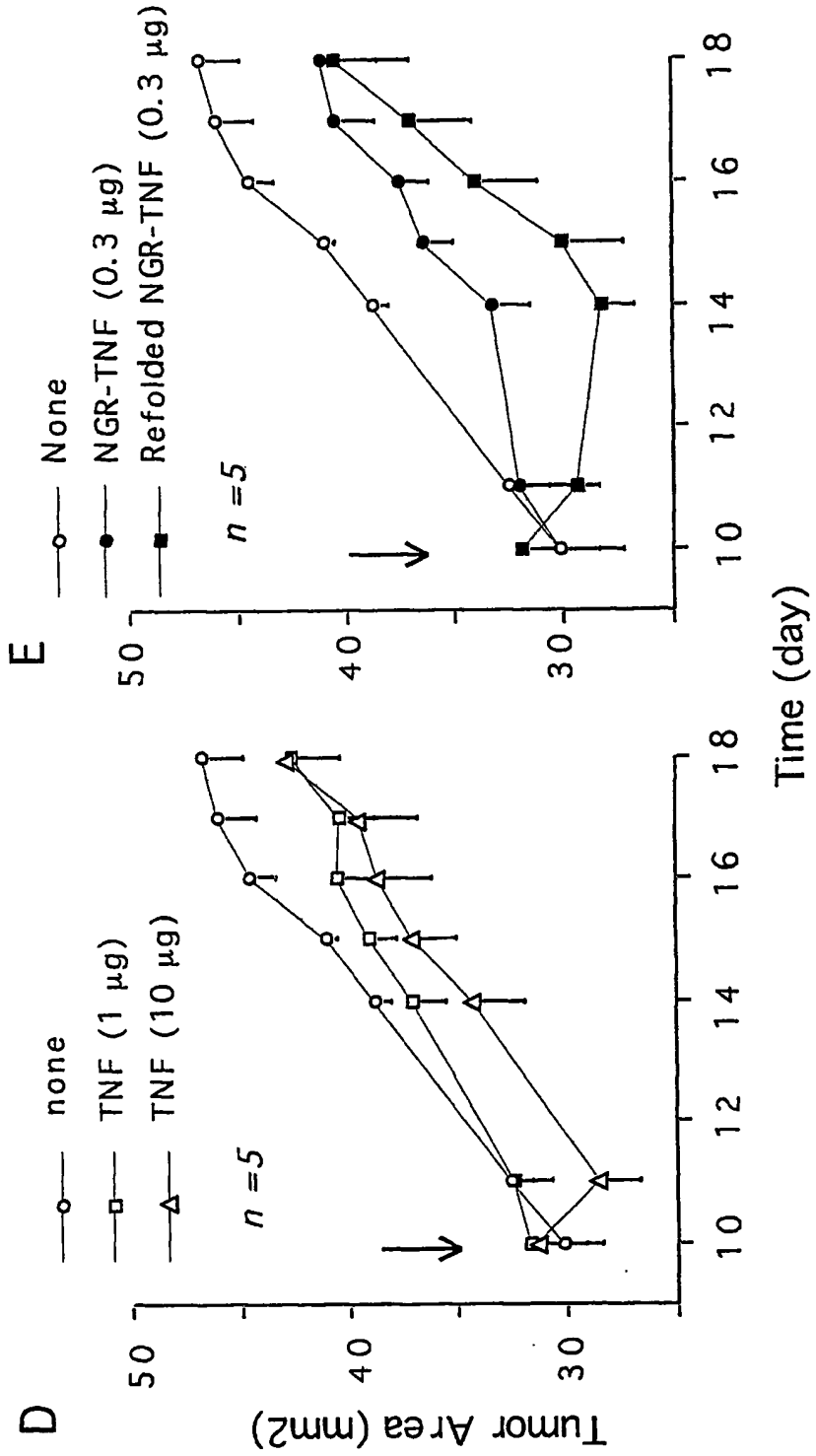

C
TNF
NGR-TNF
(non-
refolded)
n =15
Loss of weight
24 h after treatment (g)
0
- 1
- 2
P=0.001
0 1 10 1
Dose (µg)

FIGURE 4


D
E
none
TNF (1 µg)
TNF (10 µg)
None
NGR-TNF (0.3 µg)
Refolded NGR-TNF (0.3 µg)
n =5
n =5
Tumor Area (mm2)
Time (day)
50
40
30
10
12
14
16
18

SEQUENCE LISTING

<110> Fondazione Centro San Raffaele del Monte Tabor

<120> Modified cytokines for use in cancer therapy

<130> modcyt

<140>
<141>

<160> 7

<170> PatentIn Ver. 2.1

<210> 1
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 1
ctggatcctc acagagcaat gactccaaag 30

<210> 2
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 2
tgcctcacat atgctcagat catcttctc 29

<210> 3
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

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gcagatcata tgtgcaacgg ccgttgcggc ctcagatcat cttctc 46

<210> 4
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

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atatcatatg tgcaacggcc gttgcggcgt cagatcatct tctcg 45

<210> 5
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 5
tcaggatcct cacagggcaa tgatcccaaa gtagac 36

<210> 6
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 6
atatctacat atgcacggca cagtcattga aagcc 35

<210> 7
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 7
tcggatcctc agcaacggcc gttgcagccg gagcgactcc ttttccgctt
cttgaggc 58